6 DETERMINATION OF SPECIES ORIGIN

6.1 GOALS

6.1.1 To acquire a basic understanding of immunology, including the theory and procedures for species origin determination.

6.1.2 To acquire a thorough understanding of the use of controls.

6.1.3 To become acquainted with the specificity, sensitivity, and limitations of the Ouchterlony double diffusion method.

6.2 TASKS

6.2.1 Determine the sensitivity of anti-human precipitin serum by testing various dilutions in Normal Saline (neat, 1:5, 1:10, 1:20, 1:50, 1:100) of known human blood and bloodstains using Ouchterlony double diffusion.

6.2.1.1 Normal Saline (0.9%):

- 9 g Sodium chloride
- 1000 ml Distilled water
- Mix thoroughly until dissolved.

6.2.2 Test at least 15 bloodstains subjected to various environmental conditions (heat, moisture, heat and moisture combined), and decomposition using Ouchterlony double diffusion.

6.2.3 Test at least 15 bloodstains exposed to various contaminants (including, but not limited to superglue, fingerprint powder, ninhydrin, redwop power - rhodamine base, bleach, soap, motor oil, luminol, and mold) using Ouchterlony double diffusion.

6.2.4 Test the precipitating antisera in the routine species collection addressed in 6.4.6 for cross-reactivity using Ouchterlony double diffusion. Observe specificity.

6.2.5 Stain Ouchterlony plates using Coomassie Brilliant Blue R250. Note enhancement of weak reactions.

6.2.6 Instruction by and observation of qualified examiners performing routine examinations of case material.

6.2.7 Test at least 10 unknown stains provided by training coordinator or designee.

6.2.8 Read applicable literature. Refer to Appendix A and Appendix B.

6.3 TRAINING EVALUATION

6.3.1 Knowledge

6.3.1.1 Review of notes in training notebook by training coordinator.

6.3.1.2 Mini-mock trials/oral and practical examinations.

6.3.1.3 Completion of checklist by training coordinator.

6.3.2 Skills

6.3.2.1 Observation by training coordinator or designee.

6.3.2.2 Review of notes in training notebook by training coordinator.

6.3.2.3 Mini-mock trials/oral and practical examinations.

6.3.2.4 Completion of checklist by training coordinator.

6.4 SPECIES IDENTIFICATION – TECHNICAL NOTES

6.4.1 The procedure described in this section requires the use of precipitating antiserum to determine species origin. This procedure is an immunological procedure for the identification of animal or human protein. The identification is made by comparing the reaction of the unknown protein (in the case sample) against a known antiserum with the reaction of a known protein against a known antiserum.

6.4.2 A positive control (a known sample of normal serum or blood against which the antiserum is directed) and a substrate control (when available) must be run on each plate when the testing is performed on a case. If a substrate control is not available, distilled water will be used. The substrate control monitors for contaminating protein activity (which could cause a false positive reaction) in the unstained portion of the substrate as well as for contaminating protein activity in the reagent (distilled water) used for the extraction.

6.4.3 Alternatively, a human DNA quantitation method may be used to determine that a sample is of human origin.

6.4.4 Quality Control of Antiserums

6.4.4.1 Before using any new lot number of precipitating antiserum for testing casework samples, the specificity must be tested and appropriately documented in the laboratory's quality control (QC) records. Routine quality control testing will be performed at the time the antiserum is reconstituted, or if the antiserum is received in liquid form, within one week of receipt.

6.4.4.2 Anti-human serum, not anti-human hemoglobin, will be used with the procedure in this section for determining whether a sample is of human origin. Anti-human serum, as well as all animal antiserums in the "Routine Species Collection" specified below, must be tested against all available species (normal or whole serums and known bloods) in the "Routine Species Collection".

6.4.4.3 A positive control, a host control (typically normal rabbit serum or normal goat serum), and a negative control (distilled water) must be included in the specificity testing. The host control (representing the animal in which the antiserum was prepared) is used to demonstrate that the antiserum is not reacting to any proteins in the animal in which it was made.

6.4.4.4 The quality control documentation will include:

6.4.4.4.1 A diagrammatic representation of the placement of samples in the wells of the rosette on the Ouchterlony plate with the subsequent observations (i.e., precipitin lines).

6.4.4.4.2 Date of the testing.

6.4.4.4.3 Initials of the person conducting the testing.

6.4.4.4.4 Lot number, date of receipt, and manufacturer of the antiserum being tested.

6.4.4.4.5 Lot number, date of receipt, and manufacturer of the normal serums being used for the testing.

6.4.4.4.6 Results of the testing.

6.4.4.5 Once the appropriate testing has been performed on a particular lot number of antiserum, it need not be repeated for each case. If another vial of the same lot number is received on a different date, the QC testing described above must be repeated.

6.4.5 Quality Control of Normal (Whole) Serums

6.4.5.1 Before using any new lot number of normal serum for testing casework samples, the specificity must be tested and appropriately documented in the laboratory's quality control (QC) records. Routine quality control testing will be performed at the time the normal serum is reconstituted, or if the normal serum is received in liquid form, within one week of receipt.

6.4.5.2 All normal serums in the "Routine Species Collection" must be tested against all available antiserums in the "Routine Species Collection".

6.4.5.3 A positive control must be included in the specificity testing. Although normal human serum may be purchased, a straw colored dilution of known human blood may be used instead. Similarly, the use of known blood from other species may replace the purchase and use of normal serums from the species.

6.4.5.4 It is not necessary to conduct quality control testing on the known bloods. Label known bloods with the species name and date of preparation/initials of person preparing the sample. Store known bloods in the freezer.

6.4.5.5 The quality control documentation will include:

6.4.5.5.1 A diagrammatic representation of the placement of samples in the wells of the rosette on the Ouchterlony plate with the subsequent observations (i.e., precipitin lines).

6.4.5.5.2 Date of the testing.

6.4.5.5.3 Initials of the person conducting the testing.

6.4.5.5.4 Lot number, date of receipt, and manufacturer of the normal serum being tested.

6.4.5.5.5 Lot number, date of receipt, and manufacturer of the antiserums being used for the testing.

6.4.5.5.6 Results of the testing

6.4.5.6 Once the appropriate testing has been performed on a particular lot number of normal serum, it need not be repeated for each case. If another vial of the same lot number is received on a different date, the QC testing described above must be repeated.

6.4.6 Routine Species Collection

6.4.6.1 The following will be maintained in each laboratory as the "Routine Species Collection" and will undergo QC testing for specificity as outlined above:

6.4.6.1.1 Bovine antiserum and normal bovine serum or known blood

6.4.6.1.2 Swine antiserum and normal swine serum or known blood

6.4.6.1.3 Cat antiserum and normal cat serum or known blood

6.4.6.1.4 Dog antiserum and normal dog serum or known blood

6.4.6.1.5 Rabbit antiserum and normal rabbit serum or known blood

6.4.6.1.6 Sheep antiserum and normal sheep serum or known blood

6.4.6.1.7 Deer antiserum and normal deer serum or known blood

6.4.6.1.8 Human antiserum and normal human serum or known blood

6.4.6.1.9 Normal goat serum or known blood (goat antiserum is unavailable)

NOTE: It is recommended that the antiserums and normal serums listed above be reconstituted and QC tested at the time of receipt to ensure ready availability.

6.4.6.2 Other antiserums/normal serums, such as bear, rodent, fowl, horse, etc. should also be maintained for use in special cases and must also undergo QC testing as specified above. However, since these are used only in special cases, it is recommended that they not be reconstituted, aliquoted, and QC tested until it is determined that there is a specific need to do so.

6.4.7 Storage of Antiserum/Normal Serum

6.4.7.1 Small aliquots of the antiserum/normal serum will be prepared for routine use and frozen within one week of reconstitution (when antiserum is lyophilized) or upon receipt (when antiserum is liquid). All frozen aliquots have an indefinite expiration date.

6.4.7.2 A thawed aliquot may be stored refrigerated for up to 1 month. If the aliquot is to be maintained in this manner, the expiration date must be clearly marked on the vial. Otherwise, the aliquot will be immediately discarded following its use.

6.4.8 Labeling of Antiserum/Normal Serum

6.4.8.1 Labels on each aliquot will include:

6.4.8.1.1 The manufacturer

6.4.8.1.2 Type of antiserum or normal serum

6.4.8.1.3 Lot number

6.4.8.1.4 Date reconstituted/date frozen

6.4.8.1.5 Initials of the person preparing the aliquot

6.4.9 OUCHTERLONY (DOUBLE DIFFUSION) TEST (Reference 6, pp. 221-241, Appendix B)

6.4.9.1 Equipment

6.4.9.1.1 Punch

6.4.9.1.2 Aspirator

6.4.9.1.3 100 ml and 500 ml graduated cylinders

6.4.9.1.4 Balance

6.4.9.1.5 Spatula

6.4.9.1.6 Scissors

6.4.9.1.7 Tweezers

6.4.9.1.8 Hot plate or oven (37° C)

6.4.9.1.9 Incubator (optional)

6.4.9.1.10 Magnetic stir plate

6.4.9.1.11 Refrigerator (optional)

6.4.9.2 Materials

6.4.9.2.1 Petri dishes, slides, or comparable containers

6.4.9.2.2 Test tubes

6.4.9.2.3 Weigh boat or weigh paper

6.4.9.2.4 Moisture chamber

6.4.9.2.5 Disposable pipets

6.4.9.2.6 Capillary tubes

6.4.9.3 Reagents

6.4.9.3.1 Normal saline (0.9%)

6.4.9.3.2 Agarose gel (1%)

6.4.9.3.3 Distilled water

6.4.9.3.4 Antiserum

6.4.9.3.5 Normal serum or known blood (positive control)

6.4.9.4 Agarose Gel Preparation

6.4.9.4.1 Normal saline (0.9% NaCl):

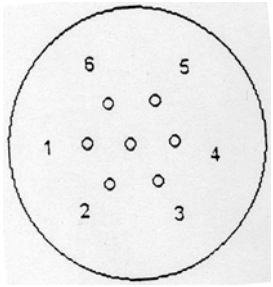
- 9 g Sodium chloride
- 1000 ml Distilled water
- Mix thoroughly until dissolved.

6.4.9.4.2 Agarose gel (1%):

- 1 g Type I agarose
- 100 ml Normal saline (0.9% NaCl)
- Heat until agarose is dissolved. Allow to cool slightly.

6.4.9.4.3 Pore the agarose into a petri dish, onto a slide, or into a comparable container to a thickness of 2-3 mm and allow to cool.

6.4.9.4.4 Cut wells in a rosette pattern (refer to the diagram below) into the gel using a punch or disposable pipet connected to an aspirator.



6.4.9.5 Storage and Labeling

6.4.9.5.1 When a batch of plates is prepared, the plates should be numbered consecutively and placed in a moisture chamber in the refrigerator. Label the moisture chamber with the lot number of the batch (date of preparation/initials of person preparing the plates).

6.4.9.5.2 There is no expiration date (see 6.4.9.6 Minimum Standards and Controls).

6.4.9.6 Minimum Standards and Controls

6.4.9.6.1 A positive control (known sample against which the antiserum is directed) and a substrate control (or if not available, distilled water) must be tested on each plate, unless the stain is on a cotton swab. It is not necessary to test submitted control swabs.

6.4.9.7 OUCHTERLONY DOUBLE DIFFUSION PROCEDURE

6.4.9.7.1 To prepare an extract of the stain, place a small cutting of the stain in distilled water until a straw color is obtained. A small piece of stained material, which is moistened with distilled water, can be used in lieu of an extract. Treat the substrate control in the same manner as the stain.

6.4.9.7.2 Add antiserum in the center well of the Ouchterlony plate with a disposable pipet or capillary tube.

6.4.9.7.3 Add appropriate extracts/pieces of stained material, the positive control, and negative control(s) to the surrounding wells. Do not overfill the wells. Avoid getting bubbles in the wells. Document the placement of samples in the wells of the rosettes on the Ouchterlony work sheet found near the end of this chapter.

6.4.9.7.4 Record the lot numbers of antiserums and normal serums used for the testing procedure.

NOTE: Alternatively, known normal serum/known blood extract may be placed in the center well with appropriate antiserums in the surrounding wells or the stain extract/piece of stained material may be placed in the center well with appropriate antiserums in the surrounding wells.

6.4.9.7.5 Incubate the plate in a moisture chamber at 37° C for 3-4 hours. Alternatively, it may be left overnight at room temperature or 4° C.

6.4.9.7.6 Record observations (precipitin lines) on the diagram, and interpret and record the results.

6.4.9.7.7 All controls must give the expected results before a conclusion can be reached on an unknown sample, i.e., white precipitin lines must be observed between the antiserum and positive control (known serum) and no precipitin lines should be observed between the antiserum and the negative control.

6.4.9.7.8 Interpretation

6.4.9.7.8.1 Positive Result = White precipitin lines between the antiserum well and the sample well

6.4.9.7.8.2 Negative Result = No precipitin lines between the antiserum well and the sample well

6.4.9.7.8.3 Inconclusive Result = Questionable precipitin lines between the antiserum well and the sample well, OR precipitin lines between the antiserum well and the negative control well(s), OR no precipitin line between the antiserum well and the positive control well. If sufficient sample remains, an inconclusive result should be repeated.

NOTE: The prozone phenomenon can result in a soluble antigen-antibody complex due to too many antibodies present to form a complete lattice (Reference 8, Appendix B). Because of this phenomenon, weak precipitin lines may be observed initially, but disappear upon staining with Coomassie Brilliant Blue R250. This is considered an inconclusive result. Therefore, it is recommended that the testing results be recorded PRIOR TO staining as well as after staining.

6.4.9.7.9 Staining the plate with Coomassie Brilliant Blue R250 may be necessary to visualize weak reactions. Refer to 6.4.10 for the Coomassie Brilliant Blue R250 staining procedure.

6.4.9.7.10 Reporting Results

6.4.9.7.10.1 Report positive test results as "(species tested according to the label on the antiserum) protein was detected…"

6.4.9.7.10.2 Report negative test results as "no (species tested according to the label on the antiserum) protein was detected…"

6.4.9.7.10.3 Report inconclusive test results as "the test for (species tested according to the label on the bottle) protein was inconclusive…"

6.4.10 COOMASSIE BRILLIANT BLUE R250 STAINING PROCEDURE (Reference 13, Appendix B)

6.4.10.1 Safety Considerations

6.4.10.1.1 Coomassie Brilliant Blue R250 - Caution! Avoid contact and inhalation! Emits toxic fumes under fire conditions! Container explosion may occur under fire conditions!

6.4.10.1.2 Methanol - Caution! Irritant! Dangerous when exposed to heat or flame!

6.4.10.1.3 Glacial acetic acid - Caution! Corrosive! Flammable!

6.4.10.2 Equipment

6.4.10.2.1 Weight

6.4.10.2.2 Oven or Incubator (40-60°C)

6.4.10.2.3 Rotator (optional)

6.4.10.2.4 10 ml, 50 ml, and 500 ml graduated cylinders

6.4.10.2.5 Balance

6.4.10.2.6 Spatula

6.4.10.2.7 Trays for staining and destaining

6.4.10.3 Materials

6.4.10.3.1 Gel bond, glass plate, or other support medium

6.4.10.3.2 Weigh boats or weigh paper

6.4.10.3.3 Whatman #1 filter paper

6.4.10.3.4 Paper towels

6.4.10.4 Reagents

6.4.10.4.1 Staining Solution

6.4.10.4.2 Destaining Solution

6.4.10.4.3 Normal saline (0.9% NaCl) – refer to 6.4.10.8.1

6.4.10.4.4 Distilled water

6.4.10.5 Preparation of Stain and Destain Solutions

6.4.10.5.1 Staining Solution

- 0.1 g Coomassie Brilliant Blue R250
- 45.0 ml Methanol
- 10.0 ml Glacial acetic acid
- 45.0 ml Distilled water
- Mix the above ingredients until thoroughly dissolved.

6.4.10.5.2 Destaining Solution

- 45.0 ml Methanol
- 10.0 ml Glacial acetic acid

- 45.0 ml Distilled water
- Mix the above ingredients until thoroughly dissolved.

6.4.10.6 Storage

6.4.10.6.1 The Staining and Destaining Solutions are stable at room temperature.

6.4.10.7 Labeling

6.4.10.7.1 Label as Staining or Destaining Solution with the lot number (date of preparation followed by the initials of the person preparing the solution).

6.4.10.7.2 There is no expiration date.

6.4.10.8 COOMASSIE BRILLIANT BLUE R250 STAINING PROCEDURE

6.4.10.8.1 Wash the plate overnight in normal saline solution (0.9% NaCl - 9 g NaCl in 1000 ml distilled water) to remove unprecipitated proteins.

6.4.10.8.1.1 If pieces of stained material were used in lieu of extracts, remove these prior to washing the plate.

6.4.10.8.1.2 The gel may detach from the plate during the washing process. Mark the orientation of the gel to ensure that it can be re-oriented properly after the washing has been completed.

6.4.10.8.1.3 Alternatively, the gel may be removed from the plate before beginning the washing process. If this is done, mark the gel to ensure that it can be re-oriented properly after the washing has been completed.

6.4.10.8.2 The next day wash the gel for approximately fifteen minutes in distilled water. Rinse the gel and repeat the wash. Ensuring proper orientation, place the gel (face up) on the hydrophilic side of a piece of gel bond or on some other support medium such as a glass plate.

6.4.10.8.3 Cover the gel with a piece of Whatman #1 filter paper moistened with distilled water. Add a layer of paper towels on top of the filter paper and press with a weight for approximately 30 minutes. Remove paper towels and filter paper and dry the gel in a 40-60° C oven.

6.4.10.8.4 Place the gel in the staining solution and allow it to soak for 1 to 10 minutes. This may be done on a rotator. Intermittently check staining progress to prevent over staining.

6.4.10.8.5 Place the gel in the destaining solution until the background is clear or until no more dye leaches from the gel. This may be done on a rotator. Change the destaining solution and destain further if desired.

6.4.10.8.6 Record observations (precipitin lines) on the diagram, and interpret and record the results. All controls must give the expected results before a conclusion can be reached on an unknown sample, i.e., blue precipitin lines must be observed between the antiserum and positive control (known serum) and no precipitin lines should be observed between the antiserum and the negative control.

6.4.10.8.7 Interpretation

6.4.10.8.7.1 Positive Result = Blue precipitin lines between the antiserum well and the sample well.

6.4.10.8.7.2 Negative Result = No precipitin lines between the antiserum well and the sample well.

6.4.10.8.7.3 Inconclusive Result = Questionable precipitin lines between the antiserum well and the sample well, OR precipitin lines between the antiserum well and the negative control well(s), OR no precipitin line observed between the antiserum well and the positive control well. If sufficient sample remains, an inconclusive result should be repeated.

6.4.10.8.8 Reporting Results

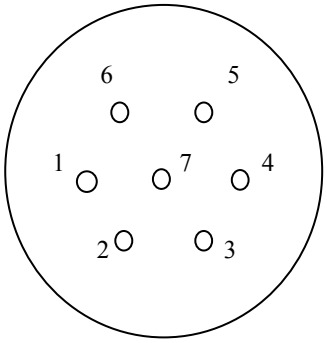
6.4.10.8.8.1 Refer to 6.4.9.7.10.

VIRGINIA DEPARTMENT OF FORENSIC SCIENCE
OUCHTERLONY FOR SPECIES DETERMINATION

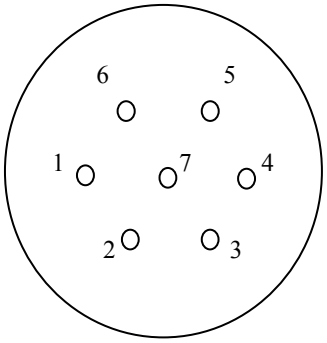
ANALYST: _____ **FS LAB#:** _____

DATE: _____

PLATE#



PLATE#



	SAMPLE	LOT# / SOURCE	RESULTS
1			
2			
3			
4			
5			
6			
7			

	SAMPLE	LOT# / SOURCE	RESULTS
1			
2			
3			
4			
5			
6			
7			

REAGENT	LOT# / SOURCE
Saline solution	
Coomassie blue staining solution	
Destaining solution	

COMMENTS: _____

DETERMINATION OF SPECIES ORIGIN STUDY QUESTIONS

1. What is an antigen?
2. What are the conditions of antigenicity?
3. What is an antibody?
4. How is antiserum made? How is normal serum made?
5. What is meant by cross-reactivity?
6. What are the advantages of Ouchterlony double diffusion? Are there disadvantages?
7. Explain Ouchterlony double diffusion and the purpose of each component/control.
8. What does a positive result look like and what does it tell you?
9. When using the Coomassie Blue stain, what is the dye staining? Why is this used?
10. What is Prozone and Postzone?
11. What would you do if you had a very small possible bloodstain and the investigator wanted to know if it was human blood, but species testing would consume the stain?
12. Why is specificity testing of antiserums and normal serums required prior to using these for testing case material?

TRAINEE CHECKLIST FOR THE DETERMINATION OF SPECIES ORIGIN

Name of Trainee:_____

1. Completion of the following tasks using Ouchterlony double diffusion:

   Tested known human blood and bloodstains of varying dilutions (neat, 1:5, 1:10, 1:20, 1:50, 1:100).

   Date:_____ Training Coordinator:_____

   Comments:_____

   Tested bloodstains subjected to various environmental conditions (15 minimum).

   Date:_____ Training Coordinator:_____

   Comments:_____

   Tested bloodstains exposed to various contaminants (15 minimum).

   Date:_____ Training Coordinator:_____

   Comments:_____

   Tested the precipitating antisera in the routine species collection addressed in 6.4.6 for cross- reactivity.

   Date:_____ Training Coordinator:_____

   Comments:_____

   Stained Ouchterlony plates using Coomassie Brialliant Blue R250.

   Date:_____ Training Coordinator:_____

   Comments:_____

   Accurately tested at least 10 unknown stains (provided by training coordinator or designee).

   Date:_____ Training Coordinator:_____

   Comments:_____

2. Trainee has developed a thorough understanding of the theory behind species origin determination, including basic immunology, specificity, sensitivity, and limitations of all methods, as well as the purpose and use of controls. An unquestionably sound technique has been developed for the use of Ouchterlony double diffusion.

   Date:_____ Training Coordinator:_____

   Comments:_____

3. Notebook is organized and complete.

   Date:_____ Training Coordinator:_____

   Comments:_____

4. Trainee has participated in a mock trial and/or practical or oral examinations. Performance was satisfactory.

   Date:_____ Training Coordinator:_____

   Comments:_____

5. Trainee has read and understands all applicable literature.

   Date:_____ Training Coordinator:_____

   Comments:_____

♦END